



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C11D 3/386	A1	(11) International Publication Number: WO 88/ 09367 (43) International Publication Date: 1 December 1988 (01.12.88)
(21) International Application Number: PCT/US88/01844 (22) International Filing Date: 31 May 1988 (31.05.88) (31) Priority Application Number: 056,500 (32) Priority Date: 29 May 1987 (29.05.87) (33) Priority Country: US (60) Parent Application or Grant (63) Related by Continuation US 056,500 (CIP) Filed on 29 May 1987 (29.05.87) (71) Applicant (for all designated States except US): GEN- ENCOR, INC. [US/US]; 180 Kimball Way, So. San Francisco, CA 94080 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : KOLATTUKUDY, Pappachan [US/US]; 2301 Hoxton Court, Columbus, OH 43220 (US). POULOSE, Ayrookaran, J. [IN/US]; 2540 Carmel Drive, San Bruno, CA 94066 (US). (74) Agent: PASSE, James, G.; Genencor, Inc., 180 Kimball Way, So. San Francisco, CA 94080 (US). (81) Designated States: AT (European patent), BE (Euro- pean patent), CH (European patent), DE (Eur pean patent), FR (European patent), GB (European pa- tent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt</i> <i>of amendments.</i>
(54) Title: CUTINASE CLEANING COMPOSITION (57) Abstract This invention relates to cleaning compositions and methods for using them. Particularly, the invention relates to compositions comprising a surfactant and a cutinase enzyme.		

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Cutinase Cleaning CompositionsBackground of the Invention

a) Field of the Invention

The invention relates to enzymatic cleaning compositions and methods for using them. Particularly the invention relates to cleaning compositions comprising a surfactant and a cutinase enzyme.

b) Background Information

A wide variety of enzymes are well known for use in cleaning compositions. The use of B. subtilis and B. licheniformis protease in commercial preparations is common. Other enzymes have also been used in commercial cleaning compositions such as, for example, U.S. Patent No. 4,011,169, and British Patent No. 1,293,613. Also a comprehensive review article of lipases in cleaning compositions can be found in Journal of Applied Biochemistry, 2:218-229 (1980) in an article entitled "Lipases as Detergent Components". Lipolytic detergent additives are also known from e.g., British Patent Specification No. 1,293,613 and Canadian Patent No. 835,343.

U.S. Patent No. 3,950,277 and British Patent Specification No. 1,442,418 disclose lipase enzymes combined with an activator and calcium and/or magnesium ions, respectively, which are utilized to pre-soak soiled fabrics and to remove triglyceride stains and soils from polyester or polyester/cotton fabric blends, respectively. Suitable microbial lipases for use therein (apart from animal and plant derived lipases) are said to be those derived from Pseudomonas, Aspergillus, Pneumococcus, Staphylococcus, and Staphylococcus toxins, Mycobacterium tuberculosis, Mycotorula lipolytica, and Sclerotinia.

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British Patent Specification No. 1,372,034 discloses a detergent composition comprising a bacterial lipase produced by Pseudomonas stutzeri strain ATCC 19154. Furthermore, it is recommended that the preferred lipolytic enzymes should have a pH optimum between 6 and 10, and should be active in said range, preferably between 7 and 9. Around 1970, this presumed Pseudomonas stutzeri strain was reclassified as Pseudomonas aeruginosa, as appears for example from the ATCC catalogues.

European Patent Application EP-A-0130064 discloses an enzymatic detergent additive comprising a lipase isolated from Fusarium oxysporum with an alleged higher lipolytic cleaning efficiency than conventional lipases.

In European Patent Application No. 0214761, Enzymatic detergent additives are described as the active component of which is a microbially produced lipase from a strain of Pseudomonas cepacia. The lipases described therein are claimed to be superior to the lipolytic detergent action of the prior art, especially at low temperature washing processes (around 60°C and below).

In PCT Patent Application No. 87/00859 other novel lipolytic enzymes are described as having an optimal pH in the range of 8 - 10.5 at a temperature of 60°C or less from bacterial strains selected from Pseudomonas pseudoalcaligenes, P. stutzeri and Acinetobacter calcoaceticus. These enzymes are described as particularly effective at low temperatures; i.e., 40°C or lower and effective in both liquid and solid detergent compositions.

Also in U.S. Patent No. 3,950,277, it is described in general terms that lipases from Pseudomonas are suited as agents for removal of oily stains from fabrics, if used together with a special group of lipase activators. The art cited does not, however, cover cutinase enzymes from Pseudomonas or any other microbial source. However, prior art enzymes for use in cleaning compositions, while effective on many proteins and lipids, are not completely effective against all stains commonly found in laundry and other cleaning applications.

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Further, many lipases are not stable at pH 8 - 11 where most cleaning compositions are used. Even further, most enzymes for use in cleaning compositions are not very stable, if at all, under oxidative conditions or in the presence of other enzymes such as proteases.

SUMMARY OF THE INVENTION

Accordingly it has been discovered that combinations of a surfactant and a substantially pure microbial cutinase enzyme are effective compositions for cleaning applications. The cutinase enzyme preparations possess activity at pH of from about 8 and 11, exhibit cleaning activity in aqueous solution at concentrations from about .05 mg/L to about 100 mg/L or more at temperatures from about 20°C to about 50°.

The enzymes are oxidatively stable and stable in the presence of other enzymes such as proteases. Even further, the cutinases show a synergistic effect when a plurality of surfactants are used with the cutinase.

The invention also relates to the improved process for enzymatically cleaning a material with an aqueous solution; the improvement comprising adding a substantially pure cutinase to the cleaning solution.

DETAILED DESCRIPTION OF THE INVENTION

Applicant has discovered that cutinase enzymes are useful when included in cleaning compositions. These compositions may take on a variety of forms such as for laundry cleaning, household and industrial cleaning, and the like. The cleaning compositions comprise combinations of known surfactants and a microbial cutinase enzyme which can be used to clean a wide variety of materials. The composition can be added to aqueous solution or solid powder, or formulated in an aqueous solution or solid powder and used according to conventional cleaning techniques.

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Enzyme

Cutinases are well known in the art and are available from a wide variety of sources. See *Cutinases from Fungi and Pollen*, P.E. Kolattukudy, pg. 472 - 504, incorporated herein by reference, for discussion of cutinases useful in the practice of the invention. A preferred cutinase is that cutinase isolated in a substantially pure form from *Pseudomonas putida*, particularly the *P. putida*, ATCC 53552, described in copending U.S. patent application, Serial No. 932,959 filed November 19, 1986 and incorporated herein by reference, which enzyme therefrom has the following amino acid sequence:

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1	ala	pro	leu	pro	asp	thr	pro	gly	ala	10	pro	phe	pro
	ala	val	ala	asn	phe	ala	arg	ser	gly	20	pro	tyr	thr
	thr	ser	ser	gln	ser	30	gly	pro	ser		cys	arg	ile
	tyr	arg	pro	40	arg	asp	leu	gly	gln	gly	val	arg	
	his	50	pro	val	ile	leu	trp	gly	asn	gly	thr	gly	60
	gly	pro	ser	thr	tyr	ala	gly	leu	leu	70	ser	his	trp
	ala	ser	his	gly	phe	val	val	ala	ala	80	ala	gln	thr
	ser	asn	ala	gly	thr	90	arg	gln	met		leu	ala	cys
	leu	asp	tyr	100	leu	val	arg	gln	asn	asp	thr	pro	tyr
	gly	110	thr	tyr	ser	gly	lys	leu	asn	thr	gly	arg	120
	gly	thr	ser	gly	his	ser	gln	gly	gly	130	gly	gly	ser
	ile	met	ala	gly	gln	asp	thr	arg	val	140	arg	thr	thr
	ala	pro	ile	gln	pro	150	thr	leu	gly		leu	gly	his
	asp	ser	ala	160	ser	gln	arg	arg	gln	gln	gly	pro	met
	phe	170	leu	met	ser	gly	gly	gly	asp	thr	ile	ala	180
	pro	tyr	leu	asn	ala	gln	pro	val	tyr	190	arg	arg	ala
	asn	val	pro	val	phe	trp	gly	gln	arg	200	arg	tyr	val
	ser	his	phe	gln	pro	210	gly	ser	gly		gly	ala	tyr
	arg	gly	pro	220	ser	thr	ala	trp	phe	arg	phe	gln	leu
	met	230	asp	asp	gln	asp	ala	arg	ala	thr	phe	tyr	240
	ala	gln	cys	ser	leu	cys	thr	ser	leu	250	leu	trp	
	ser	val	gly	arg	arg	gly	leu						

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Other sources of bacterial and fungal cutinases include:

Fusarium solani pisi

Fusarium roseum sambucinum

Fusarium roseum culmorum

Helminthosporium sativum

Ulocladium consortiale

Streptomyces scabies

Colletotrichum capsici

Phytophthora cactorum

Botrytis cineria

Colletotrichum gloeosporioides

The cutinase of the invention should preferably be selected to cause at least about 10%, and preferably 20%, hydrolysis of the given fat under given conditions. Normally the amount would be in a concentration of from about .01% to about 5.0% by weight of the surfactant, and preferably from about .05% to about 3%, such that upon dilution in wash water it is in a concentration of at least about .05mg/L. Further, one skilled in the art could take the preferred cutinase or, for that matter, any cutinase of the invention or any immunologically identical cutinase and use random or selective replacement of amino acids to produce other cutinases which are more or less selective toward given substrates or include modification in activity such as oxidative stability. Substantially pure cutinase includes the isolated enzyme as well as the broth containing the enzyme in unpurified form but essentially free of other enzymes and enzyme sources.

The natural substrate of cutinase is cutin which is a biopolyester polymer which covers the plant leaves, fruits, etc., see Structure, Biosynthesis and Biodegradation of Cutin and Suberin. (1981), P.E. Kolattukudy, Ann. Rev. Plant Physiol., 32, pgs. 539-567. Stains comprising lipids which could be hydrolyzed or bound by cutinase on a substrate such as cloth would be similar to the natural substrate cutin. Cutinase, for these types of stains, will be more

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ffective than the prior art lipases. The cutinases will work especially well on gravy, oils and greases, plant or grass, oil based makeup and collar stains.

Cutinases are distinguishable from other lipases by methods well known in the art, See R.E. Purdy and P.E. Kolattukudy, Biochemistry, "Cutinase Assay", 14:2831-2840, (1975). Microbial cutinases from both fungal and bacterial sources have very good activity at pH 8 - pH 11 which is an ideal pH condition for detergent use.

Because of the specific activity of cutinases, it is a preferred aspect of the invention to combine one or more other cutinases, or one or more other enzymes, such as proteases, amylases or other lipases, along with the cutinase of the invention in the cleaning composition. Further, Applicant shows a synergistic increase in hydrolytic activity of cutinase when two or more surfactants are combined along with the cutinase enzyme.

Cutinases then are ideal for cleaning composition inclusion. They have stability oxidatively such as in H_2O_2 . They have good stability in a temperature range of from about 20 - 50°C which is ideal from a cleaning point of view. They are also stable in the presence of other enzymes; e.g., proteases, and as such, are ideal for mixtures of enzymes.

The Surfactant

A number of known compounds are suitable surfactants useful in the present compositions. These include nonionic, anionic, cationic, or zwitterionic detergents, as disclosed in U.S. 4,404,128 to Barry J. Anderson and U.S. 4,261,868 to Jiri Hora et al. The art is familiar with the different formulations which can be used as cleaning compositions.

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The Cleaning Compositions and Method of Use

Cutinases can be formulated as a purposefully added ingredient into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about 0.01 to about 5% (preferably .05 to .5%) by weight of the detergent. These detergent cleaning compositions can also include other enzymes such as known proteases and amylases, as well as bleaches, colorants, builders, and stabilizers.

The cutinase of the invention may be added to powdered detergents in the form of granulates or prills, prepared by methods known in the art such as described in British Patent Nos. 1,324,116 and 1,362,365 and U.S. patent Nos. 3,519,570; 4,106,991 and 4,242,219.

The cutinase preparations of the invention can be prepared by cultivating the microorganisms defined herein or otherwise cutinase containing microorganism under appropriate conditions. In order to obtain reasonable yields of enzyme, media containing readily assimilable carbon and energy sources as necessary such as a nitrogen source, as well as calcium and magnesium salts and trace elements and cutin, or monomers of cutin, or compounds resembling cutin or cutin monomers. One could also obtain the gene for cutinase and express in any organism of choice where one may not have to add cutin or cutin monomers into the fermentation.

The addition of cutinase to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for detergent compositions containing enzymes is also suitable for the present compositions.

Although the preferred form of the invention has been described above, it will be obvious to those skilled in the art to which the invention pertains, that, after understanding the invention and in view of the following testing as a whole, various changes and equivalent modifications may be made without parting from the scope of the invention as defined by the claims.

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STABILITY CUTINASE AGAINST PROTEASES

Reaction conditions:

Buffer: 0.1 M NaP, pH 10

Temp: 37°C

Lipase: 42 ug/ml

Approximately 1:1 protease:cutinase aqueous solution were made up with the following results.

ENZYME ACTIVITY

Protease

Incubation Time	0 min	5 min	10 min	15 min	14 hrs
None	1.57	1.60	1.47	1.63	1.61
Maxacal (35ug/ml)	1.68	1.58	1.72	1.66	0.134
Esperase	1.73	1.64	1.59	1.51	0.456
(64 ug/ml)					

Maxical is Gist-Brocade's brand of subtilisin enzyme (protease)

Esperase is Novo's brand of protease enzyme (protease)

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TEMPERATURE STABILITY OF BACTERIAL CUTINASE

HALF LIFE AT 50°C

<u>pH</u>	<u>Hrs.</u>
7	30
8	25
9	12
10	0.3

Enzyme was incubated at 50°C in 0.1M sodium phosphate buffer at various pH's and activity was measured by hydrolysis of trioctanoin in polyvinyl alcohol emulsions.

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EFFECT OF DETERGENTS ON HYDROLASE ACTIVITY

Reaction Conditions:

substrate: p-nitro-phenyl butyrate, 1mM (pnp)

pH: 8.0

buffer: 0.1M tris pH 8.0

temperature: 25°C

enzyme: bacterial cutinase from ATCC 53552

An aqueous solution with the following were made up and the enzyme activity was measured in these solutions using pnp as a substrate by following absorbance of p-nitrophenol at 410 nm.

<u>Triton x100%</u>	<u>SDS %</u>	<u>% Activity</u>
0	0	100
0.2	—	78
0.4	—	60
—	0.05	30
—	0.1	23
—	0.2	14
—	0.4	6
0.4	0.4	78
0.2	0.2	98
0.2	0.05	125
0.2	0.1	138

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6.1 0.1 130

0.05 0.05 132

- 1) Non-ionic detergent inhibition is not significant at low concentrations.
- 2) An ionic detergent inhibitor at high concentrations.
- 3) Mixture of anionic and non-ionic detergents stimulate activity.

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STABILITY TOWARD OXIDANTS

Cutinase .5 mg/ml in 0.1M sodium phosphate buffer, was incubated with various levels of hydrogen peroxide at pH 8.4 and 25°C for 2 hours, and hydrolytic activity was measured by a pH-stat using trioctanol-polyvinyl alcohol emulsion.

[H₂O₂ ppm] Hydrolytic Activity

% Remaining

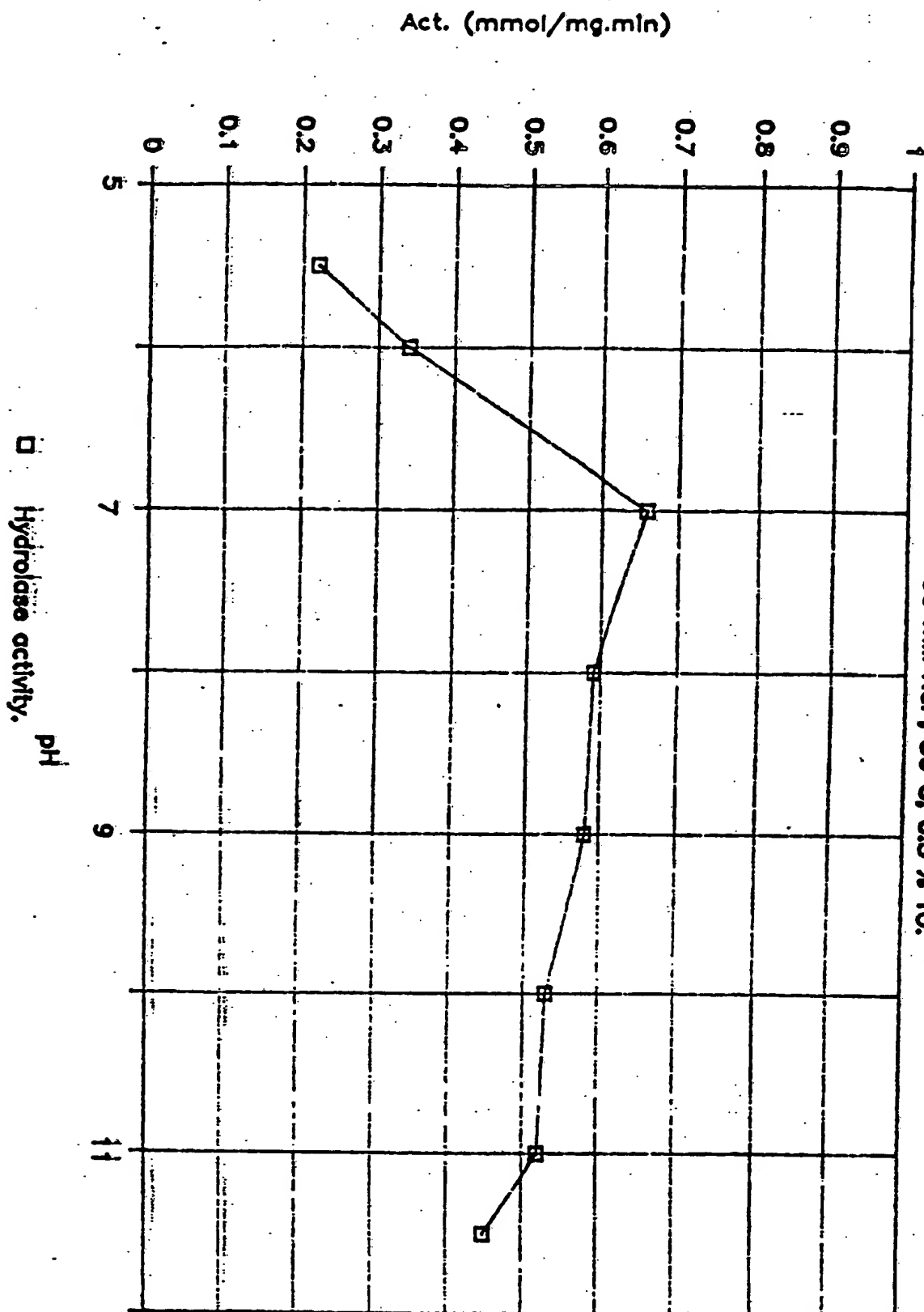
0	100
100	86
200	86
500	91
1000	95

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TABLE 1

pH—optimum of bacterial cutinase.

50 mM NaP, 30 °C, 0.5 % TO.



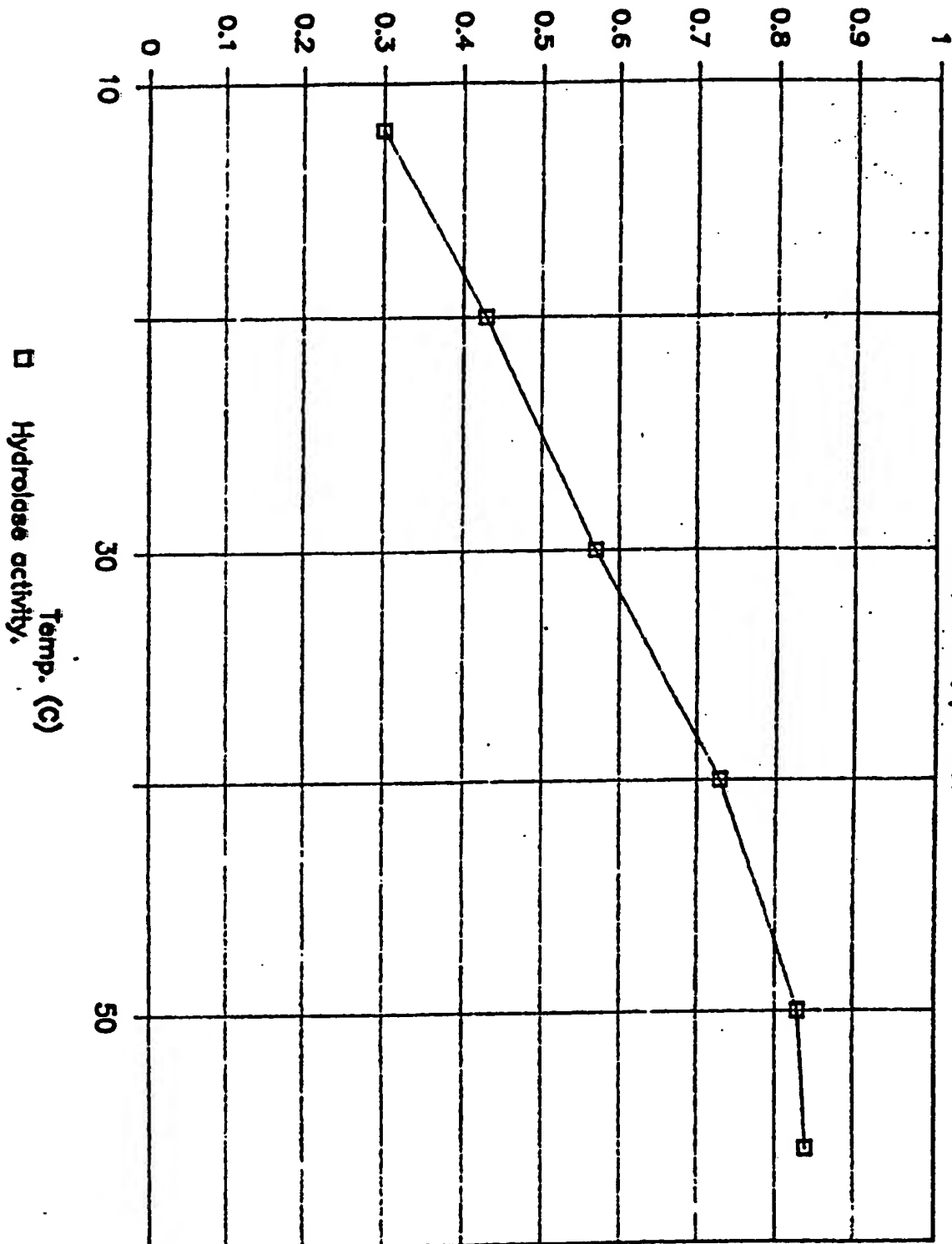
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Act. (mm l/mg.min)

TABLE 2

Temperature optimum of bact. cutinase.

50 mM NaP, pH 8.0.



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What is Claimed is:

1. An enzymatic cleaning composition comprising a substantially pure microbial cutinase in an amount sufficient that upon dilution in an aqueous solution for use there is sufficient cutinase to achieve a concentration of from about .05 mg/L to about 100 mg/L of cutinase.
2. A composition according to Claim 1 wherein the cutinase is the cutinase present in an amount of from about 0.01% to about 5% by weight of the surfactant.
3. A composition according to Claim 2 wherein the Pseudomonas putida is ATCC 53552.
4. A composition according to Claim 1 which further comprises one or more enzymes selected from lipases, amylases or proteases.
5. A composition according to Claim 1 wherein the cutinase selected is capable of causing at least 10% hydrolysis of a cutin or cutin-like substrate desirous of being cleaned from a given surface.
6. A composition according to Claim 1 wherein the cutinase is a non-naturally occurring cutinase having at least one amino acid randomly or selectively replaced by an amino acid not naturally found at that position.

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7. A composition according to Claim 1 wherein the cutinase is added in the form of cell fermentation broth containing cutinase but essentially free of other enzymes or enzyme sources.
8. An enzymatic cleaning additive wherein the active component which is a substantially pure microbially produced cutinase.
9. A composition according to Claim 8 which further comprises one or more enzymes selected from lipases, amylases or proteases.
10. A composition according to Claim 8 comprising a plurality of surfactants.
11. A composition according to Claim 10 wherein the surfactants are SDS and Triton X-100.
12. An improved method for enzymatically cleaning a material having a cutin or cutin-like stain comprising:
 - a) selecting a cutinase enzyme;
 - b) forming an aqueous solution with said enzyme in a concentration of from about 0.05 mg/L to about 100 mg/L;
 - c) contacting the material with the solution of step b); and
 - d) rinsing the material of step c).
13. A method according to Claim 12 wherein the aqueous solution further comprises a surfactant compatible with the selected cutinase.

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14. A method according to Claim 13 wherein the aqueous solution further comprises a surfactant compatible with the selected cutinase.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 88/01844

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 11 D 3/386								
II. FIELDS SEARCHED <div style="text-align: right; margin-right: 100px;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 25%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; vertical-align: top;">IPC⁴</td> <td style="border: none; vertical-align: top;">C 11 D; C 12 N</td> </tr> </table> <div style="text-align: center; margin-top: 10px;"> <small>Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸</small> </div>			Classification System	Classification Symbols	IPC ⁴	C 11 D; C 12 N		
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III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: left;">Category ⁹</th> <th style="width: 60%; text-align: left;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; text-align: left;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">P, X</td> <td style="vertical-align: top;"> EP, A, 0268452 (GENENCOR) 25 May 1988 see example 13; abstract; page 1, lines 29-30 cited in the application <div style="text-align: center; margin-top: 20px;">-----</div> </td> <td style="vertical-align: top;"> 1-3, 5, 6, 8, 12, 13 </td> </tr> </tbody> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	P, X	EP, A, 0268452 (GENENCOR) 25 May 1988 see example 13; abstract; page 1, lines 29-30 cited in the application <div style="text-align: center; margin-top: 20px;">-----</div>	1-3, 5, 6, 8, 12, 13
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><small>* Special categories of cited documents: ¹⁰</small></p> <p><small>"A" document defining the general state of the art which is not considered to be of particular relevance</small></p> <p><small>"E" earlier document but published on or after the international filing date</small></p> <p><small>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</small></p> <p><small>"O" document referring to an oral disclosure, use, exhibition or other means</small></p> <p><small>"P" document published prior to the international filing date but later than the priority date claimed</small></p> </div> <div style="width: 45%;"> <p><small>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</small></p> <p><small>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</small></p> <p><small>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</small></p> <p><small>"A" document member of the same patent family</small></p> </div> </div>								
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> Date of the Actual Completion of the International Search 29th August 1988 </td> <td style="width: 50%; border: none; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: right; font-size: 1.2em;">20 SEP 1988</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="border: none; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: right;"> P.C.G. VAN DER PUTTEN </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 29th August 1988	Date of Mailing of this International Search Report <div style="text-align: right; font-size: 1.2em;">20 SEP 1988</div>	International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: right;"> P.C.G. VAN DER PUTTEN </div>		
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